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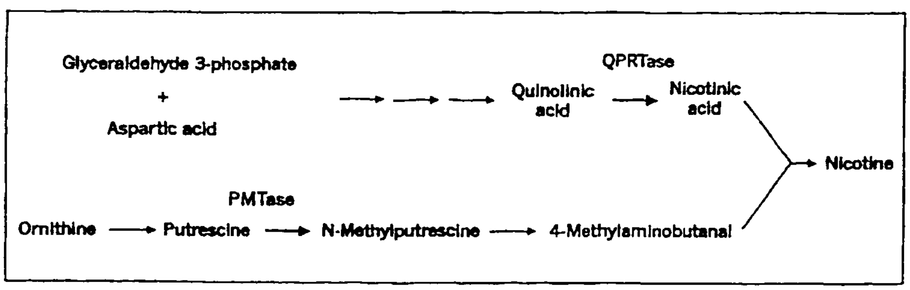

(57) Abstract

DNA encoding a plant quinolate phosphoribosyl transferase (QPRTase) enzyme, and constructs comprising such DNA are provided. Methods of altering quinolate phosphoribosyl transferase expression are provided.

REGULATION OF QUINOLATE PHOSPHORIBOSYL TRANSFERASE EXPRESSION

FEDERALLY SPONSORED RESEARCH

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FIELD OF THE INVENTION

This invention relates to plant quinolate phosphoribosyl transferase (QPRTase) and to DNA encoding this enzyme. In particular, this invention relates to the use of DNA encoding quinolate phosphoribosyl transferase to produce transgenic plants having genetically altered nicotine levels, and the plants so produced.

BACKGROUND OF THE INVENTION

The production of tobacco with decreased levels of nicotine is of interest, given concerns regarding the addictive nature of nicotine. Additionally, tobacco plants with extremely low levels of nicotine production, or no nicotine production, are attractive as recipients for transgenes expressing commercially valuable products such as pharmaceuticals, cosmetic components, or food additives. Various processes have been designed for the removal of nicotine from tobacco. However, most of these processes remove other ingredients from tobacco in addition to nicotine, thereby adversely affecting the tobacco. Classical crop breeding techniques have produced tobacco plants with lower levels of nicotine (approximately 8%) than that found in wild-type tobacco plants. Tobacco plants and tobacco having even further reductions in nicotine content are desirable.

One approach for reducing the level of a biological product is to reduce the amount of a required enzyme in the biosynthetic pathway leading to that product. Where the affected enzyme naturally occurs in a rate-limiting amount (relative to the other enzymes required in the pathway), any reduction in that enzyme's abundance will decrease the production of the end product. If the amount of the enzyme is not normally rate limiting, its presence in a cell must be reduced to rate-limiting levels in order to diminish the pathway's output. Conversely, if the naturally-occurring amount of enzyme is rate limiting, then any increase in the enzyme's activity will result in an increase in the biosynthetic pathway's end product.

Nicotine is formed primarily in the roots of the tobacco plant and is subsequently transported to the leaves, where it is stored (Tso, *Physiology and Biochemistry of Tobacco Plants*, pp. 233-34, Dowden, Hutchinson & Ross, Stroudsburg, Pa. (1972)). An obligatory step in nicotine biosynthesis is the formation of nicotinic acid from quinolinic acid, which step is catalyzed by the enzyme quinoline phosphoribosyl transferase ("QPRTase"). QPRTase appears to be a rate-limiting enzyme in the pathway supplying nicotinic acid for nicotine synthesis in tobacco. *See, e.g.*, Feth et al., "Regulation in Tobacco Callus of Enzyme Activities of the Nicotine Pathway", *Planta*, 168, pp. 402-07 (1986); Wagner et al., "The Regulation of Enzyme Activities of the Nicotine Pathway in Tobacco", *Physiol. Plant.*, 68, pp. 667-72 (1986). The modification of nicotine levels in tobacco plants by antisense regulation of putrescence methyl transferase (PMTase) expression is proposed in US Patents 5,369,023 and 5,260,205 to Nakatani and Malik. PCT application WO 94/28142 to Wahad and Malik describes DNA encoding PMT and the use of sense and antisense PMT constructs.

A further aspect of the present invention is a method of producing a tobacco plant having decreased levels of nicotine in leaves of the tobacco plant by growing a tobacco plant with cells that comprise an exogenous DNA sequence, where a transcribed strand of the exogenous DNA sequence is complementary to endogenous quinolate phosphoribosyl transferase messenger RNA in the cells.

A further aspect of the present invention is a method of making a transgenic plant cell having increased quinolate phosphoribosyl transferase (QPRTase) expression, by transforming a plant cell known to express quinolate phosphoribosyl transferase with an exogenous DNA construct which comprises a DNA sequence encoding quinolate phosphoribosyl transferase.

A further aspect of the present invention is a transgenic *Nicotiana* plant having increased quinolate phosphoribosyl transferase (QPRTase) expression, where cells of the transgenic plant comprise an exogenous DNA sequence encoding a plant quinolate phosphoribosyl transferase.

A further aspect of the present invention is a method for increasing expression of a quinolate phosphoribosyl transferase gene in a plant cell, by growing a plant cell transformed to contain exogenous DNA encoding quinolate phosphoribosyl transferase.

A further aspect of the present invention is a method of producing a tobacco plant having increased levels of nicotine in the leaves, by growing a tobacco plant having cells that contain an exogenous DNA sequence that encodes quinolate phosphoribosyl transferase functional in the cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the biosynthetic pathway leading to nicotine. Enzyme activities known to be regulated by *Nic1* and *Nic2* are QPRTase (quinolate phosphoribosyl transferase) and PMTase (putrescence methyl-transferase).

Figure 2A provides the nucleic acid sequence of *NtQPT1* cDNA (**SEQ ID NO:1**), with the coding sequence (SEQ ID NO:3) shown in capital letters.

Figure 2B provides the deduced amino acid sequence (**SEQ ID NO:2**) of the tobacco QPRTase encoded by *NtQPT1* cDNA.

Figure 3 aligns the deduced NtQPT1 amino acid sequence and related sequences of *Rhodospirillum rubrum, Mycobacterium lepre, Salmonella typhimurium, Escherichia coli,* human, and *Saccharomyces cerevisiae.*

Figure 4 shows the results of complementation of an *Escherichia coli* mutant lacking quinolate phosphoribosyl transferase (TH265) with *NtQPT1* cDNA. Cells were transformed with an expression vector carrying *NtQPT1*; growth of transformed TH265 cells expressing *NtQPT1* on minimal medium lacking nicotinic acid demonstrated that *NtQPT1* encodes QPRTase.

Figure 5 compares nicotine levels and the relative steady-state *NtQTP1* mRNA levels in *Nic1* and *Nic2* tobacco mutants: wild-type Burley 21 (*Nic1/Nic1 Nic2/Nic2*); *Nic1*$^-$ Burley 21 (*nic1/nic1 Nic2/Nic2*); *Nic2*$^-$ Burley 21 (*Nic1/Nic1 nic2/nic2*); and *Nic1*$^-$*Nic2*$^-$ Burley 21 (*nic1/nic1 nic2/nic2*). Solid bars indicate mRNA transcript levels; hatched bars indicate nicotine levels.

Figure 6 charts the relative levels of *NtQPT1* mRNA over time in topped tobacco plants compared to non-topped control plants. Solid bars indicate mRNA transcript levels; hatched bars indicate nicotine levels.

DETAILED DESCRIPTION OF THE INVENTION

Nicotine is produced in tobacco plants by the condensation of nicotinic acid and 4-methylaminobutanal. The biosynthetic pathway resulting in nicotine production is illustrated in **Figure 1**. Two regulatory loci (*Nic1* and *Nic2*) act as co-dominant regulators of nicotine production. Enzyme analyses of roots of single and double *Nic* mutants show that the activities of two enzymes, quinolate phosphoribosyl transferase (QPRTase) and putrescence methyl transferase (PMTase), are directly proportional to levels of nicotine biosynthesis. A comparison of enzyme activity in tobacco tissues (root and callus) with different capacities for nicotine synthesis shows that QPRTase activity is strictly correlated with nicotine content (Wagner and Wagner, *Planta* 165:532 (1985)). Saunders and Bush (*Plant Physiol* 64:236 (1979) showed that the level of QPRTase in the roots of low nicotine mutants is proportional to the levels of nicotine in the leaves.

The present invention encompasses a novel cDNA sequence (**SEQ ID NO:1**) encoding a plant quinolate phosphoribosyl transferase (QPRTase) of **SEQ ID NO:2**. As QPRTase activity is strictly correlated with nicotine content, construction of transgenic tobacco plants in which QPRTase levels are lowered in the plant roots (compared to levels in wild-type plants) result in plants having reduced levels of nicotine in the leaves. The present invention provides methods and nucleic acid constructs for producing such transgenic plants, as well as such transgenic plants. Such methods include the expression of antisense *NtQPT1* RNA, which lowers the amount of QPRTase in tobacco roots. Nicotine has additionally been found in non-tobacco species and families of plants, though the amount present is usually much lower than in *N. tabacum*.

The present invention also provides sense and antisense recombinant DNA molecules encoding QPRTase or QPRTase antisense RNA molecules, and vectors comprising those recombinant DNA molecules, as well as transgenic plant cells and plants transformed with those DNA molecules and vectors. Transgenic tobacco cells and plants of this invention are characterized by lower or higher nicotine content than untransformed control tobacco cells and plants.

Tobacco plants with extremely low levels of nicotine production, or no nicotine production, are attractive as recipients for transgenes expressing commercially valuable products such as pharmaceuticals, cosmetic components, or food additives. Tobacco is attractive as a recipient plant for a transgene encoding a desirable product, as tobacco is easily genetically engineered and produces a very large biomass per acre; tobacco plants with reduced resources devoted to nicotine production accordingly will have more resources available for production of transgene products. Methods of transforming tobacco with transgenes producing desired products are known in the art; any suitable technique may be utilized with the low nicotine tobacco plants of the present invention.

Tobacco plants according to the present invention with reduced QPRTase expression and reduced nicotine levels will be desirable in the production of tobacco products having reduced nicotine content. Tobacco plants according to the present invention will be suitable for use in any traditional tobacco product, including but not limited to pipe, cigar and cigarette tobacco, and chewing tobacco, and may be in any form including leaf tobacco, shredded tobacco, or cut tobacco.

The constructs of the present invention may also be useful in providing transgenic plants having increased QPRTase expression and increased nicotine content in the plant. Such constructs, methods using these constructs and the plants so produced may be desirable in the production of tobacco products having altered nicotine content, or in the production of plants having nicotine content increased for its insecticidal effects.

The present inventors have discovered that the *TobRD2* gene (*see* Conkling et al., *Plant Phys.* 93, 1203 (1990)) encodes a *Nicotiana tabacum* QPRTase, and provide herein the cDNA sequence of *NtQPT1* (formerly termed *TobRD2*) and the amino acid sequence of the encoded enzyme. Comparisons of the *NtQPT1* amino acid sequence with the GenBank database reveal limited sequence similarity to bacterial proteins that encode quinolate phosphoribosyl transferase (QPRTase) (**Figure 3**).

Quinolate phosphoribosyl transferase is required for *de novo* nicotine adenine dinucleotide (NAD) biosynthesis in both prokaryotes and eukaryotes. In tobacco, high levels of QPRTase are detected in roots, but not in leaves. To determine that *NtQPT1* encoded QPRTase, the present inventors utilized *Escherichia coli* bacterial strain (TH265), a mutant lacking in quinolate phosphoribosyl transferase (*nadC*$^-$). This mutant cannot grow on minimal medium lacking nicotinic acid. However, expression of the NtQPT1 protein in this bacterial strain conferred the *NadC*[+] phenotype (**Figure 4**), confirming that *NtQPT1* encodes QPRTase.

The present inventors examined the effects of *Nic1* and *Nic2* mutants in tobacco, and the effects of topping tobacco plants, on *NtQPT1* steady-state mRNA levels and nicotine levels. (Removal of apical dominance by topping at onset of flowering is well known to result in increased levels of nicotine biosynthesis and transport in tobacco, and is a standard practice in tobacco production.) If *NtQPT1* is in fact involved in nicotine biosynthesis, it would be expected that (1) *NtQPT1* mRNA levels would be lower in *Nic1/Nic2* double mutants and (2) *NtQPT1* mRNA levels would increase after topping. *NtQPT1* mRNA levels in *Nic1/Nic2* double mutants were found to be approximately 25% that of wild-type (**Figure 5**). Further, within six hours of topping, the *NtQPT1* mRNA levels in tobacco plants increased about eight-fold. Therefore, *NtQPT1* was determined to be a key regulatory gene in the nicotine biosynthetic pathway.

Transgenic Plant Cells and Plants

Regulation of gene expression in plant cell genomes can be achieved by integration of heterologous DNA under the transcriptional control of a promoter which is functional in the host, and in which the transcribed strand of heterologous DNA is complementary to the strand of DNA that is transcribed from the endogenous gene to be regulated. The introduced DNA, referred to as antisense DNA, provides an RNA sequence which is complementary to naturally produced (endogenous) mRNAs and which inhibits expression of the endogenous mRNA. The mechanism of such gene expression regulation by antisense is not completely understood. While not wishing to be held to any single theory, it is noted that one theory of antisense regulation proposes that transcription of antisense DNA produces RNA molecules which bind to and prevent or inhibit transcription of endogenous mRNA molecules.

In the methods of the present invention, the antisense product may be complementary to coding or non-coding (or both) portions of naturally occurring target RNA. The antisense construction may be introduced into the plant cells in any suitable manner, and may be integrated into the plant genome for inducible or constitutive transcription of the antisense sequence. *See, e.g.*, US Patent Nos. 5,453,566 and 5,107,065 to Shewmaker et al. (incorporated by reference herein in their entirety).

As used herein, exogenous or heterologous DNA (or RNA) refers to DNA (or RNA) which has been introduced into a cell (or the cell's ancestor) through the efforts of humans. Such heterologous DNA may be a copy of a sequence which is naturally found in the cell being transformed, or fragments thereof.

To produce a tobacco plant having decreased QPRTase levels, and thus lower nicotine content, than an untransformed control tobacco plant, a tobacco cell may be transformed with an exogenous QPRT antisense transcriptional unit comprising a partial QPRT cDNA sequence, a full-length QPRT cDNA sequence, a partial QPRT chromosomal sequence, or a full-length QPRT chromosomal sequence, in the antisense orientation with appropriate operably linked regulatory sequences. Appropriate regulatory sequences include a transcription initiation sequence ("promoter") operable in the plant being transformed, and a polyadenylation/transcription termination sequence. Standard techniques, such as restriction mapping, Southern blot hybridization, and nucleotide sequence analysis, are then employed to identify clones bearing QPRTase sequences in the antisense orientation, operably linked to the regulatory sequences. Tobacco plants are then regenerated from successfully transformed cells. It is most preferred that the antisense sequence utilized be complementary to the endogenous sequence, however, minor variations in the exogenous and endogenous sequences may be tolerated. It is preferred that the antisense DNA sequence be of sufficient sequence similarity that it is capable of binding to the endogenous sequence in the cell to be regulated, under stringent conditions as described below.

Antisense technology has been employed in several laboratories to create transgenic plants characterized by lower than normal amounts of specific enzymes. For example, plants with lowered levels of chalcone synthase, an enzyme of a flower pigment biosynthetic pathway, have been produced by inserting a chalcone synthase antisense gene into the genome of tobacco and petunia. These transgenic tobacco and petunia plants produce flowers with lighter than normal coloration (Van der Krol et al., "An Anti-Sense Chalcone Synthase Gene in Transgenic Plants Inhibits Flower Pigmentation", *Nature*, 333, pp. 866-69 (1988)). Antisense RNA technology has also been successfully employed to inhibit production of the enzyme polygalacturonase in tomatoes (Smith et al., "Antisense RNA Inhibition of Polygalacturonase Gene Expression in Transgenic Tomatoes", *Nature*, 334, pp. 724-26 (1988); Sheehy et al., "Reduction of Polygalacturonase Activity in Tomato Fruit by Antisense RNA", *Proc. Natl. Acad. Sci. USA*, 85, pp. 8805-09 (1988)), and the small subunit of the enzyme ribulose bisphosphate carboxylase in tobacco (Rodermel et al., "Nuclear-Organelle Interactions: Nuclear Antisense Gene Inhibits Ribulose Bisphosphate Carboxylase Enzyme Levels in Transformed Tobacco Plants", *Cell*, 55, pp. 673-81 (1988)). Alternatively, transgenic plants characterized by greater than normal amounts of a given enzyme may be created by transforming the plants with the gene for that enzyme in the sense (i.e., normal) orientation. Levels of nicotine in the transgenic tobacco plants of the present invention can be detected by standard nicotine assays. Transformed plants in which the level of QPRTase is reduced compared to untransformed control plants will accordingly have a reduced nicotine level compared to the control; transformed plants in which the level of QPRTase is increased compared to untransformed control plants will accordingly have an increased nicotine level compared to the control.

The heterologous sequence utilized in the antisense methods of the present invention may be selected so as to produce an RNA product complementary to the entire QPRTase mRNA sequence, or to a portion thereof. The sequence may be complementary to any contiguous sequence of the natural messenger RNA, that is, it may be complementary to the endogenous mRNA sequence proximal to the 5'-terminus or capping site, downstream from the capping site, between the capping site and the initiation codon and may cover all or only a portion of the non-coding region, may bridge the non-coding and coding region, be complementary to all or part of the coding region, complementary to the 3'-terminus of the coding region, or complementary to the 3'-untranslated region of the mRNA. Suitable antisense sequences may be from at least about 13 to about 15 nucleotides, at least about 16 to about 21 nucleotides, at least about 20 nucleotides, at least about 30 nucleotides, at least about 50 nucleotides, at least about 75 nucleotides, at least about 100 nucleotides, at least about 125 nucleotides, at least about 150 nucleotides, at least about 200 nucleotides, or more. In addition, the sequences may be extended or shortened on the 3' or 5' ends thereof.

The particular anti-sense sequence and the length of the anti-sense sequence will vary depending upon the degree of inhibition desired, the stability of the anti-sense sequence, and the like. One of skill in the art will be guided in the selection of appropriate QPRTase antisense sequences using techniques available in the art and the information provided herein. With reference to **Figure 2A** and **SEQ ID NO:1** herein, an oligonucleotide of the invention may be a continuous fragment of the QPRTase cDNA sequence in antisense orientation, of any length that is sufficient to achieve the desired effects when transformed into a recipient plant cell.

The present invention may also be used in methods of sense co-suppression of nicotine production. Sense DNAs employed in carrying out the present invention are of a length sufficient to, when expressed in a plant cell, suppress the native expression of the plant QPRTase protein as described herein in that plant cell. Such sense DNAs may be essentially an entire genomic or complementary DNA encoding the QPRTase enzyme, or a fragment thereof, with such fragments typically being at least 15 nucleotides in length. Methods of ascertaining the length of sense DNA that results in suppression of the expression of a native gene in a cell are available to those skilled in the art.

In an alternate embodiment of the present invention, *Nicotiana* plant cells are transformed with a DNA construct containing a DNA segment encoding an enzymatic RNA molecule (*i.e.*, a "ribozyme"), which enzymatic RNA molecule is directed against (*i.e.*, cleaves) the mRNA transcript of DNA encoding plant QPRTase as described herein. Ribozymes contain substrate binding domains that bind to accessible regions of the target mRNA, and domains that catalyze the cleavage of RNA, preventing translation and protein production. The binding domains may comprise antisense sequences complementary to the target mRNA sequence; the catalytic motif may be a hammerhead motif or other motifs, such as the hairpin motif. Ribozyme cleavage sites within an RNA target may initially be identified by scanning the target molecule for ribozyme cleavage sites (e.g., GUA, GUU or GUC sequences). Once identified, short RNA sequences of 15, 20, 30 or more ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complimentary oligonucleotides, using ribonuclease protection assays as are known in the art. DNA encoding enzymatic RNA molecules may be produced in accordance with known techniques. *See, e.g.*, T. Cech et al., U.S. Patent No. 4,987,071; Keene et al., US Patent No. 5,559,021; Donson et al., US Patent No. 5,589,367; Torrence et al., US Patent No. 5,583,032; Joyce, US Patent No. 5,580,967; Gold et al. US Patent No. 5,595,877; Wagner et al., US Patent No. 5,591,601; and US Patent No. 5,622,854 (the disclosures of which are to be incorporated herein by reference in their entirety). Production of such an enzymatic RNA molecule in a plant cell and disruption of QPRTase protein production reduces QPRTase activity in plant cells in essentially the same manner as production of an antisense RNA molecule: that is, by disrupting translation of mRNA in the cell which produces the enzyme. The term 'ribozyme' is used herein to describe an RNA-containing nucleic acid that functions as an enzyme (such as an endoribonuclease), and may be used interchangeably with 'enzymatic RNA molecule'. The present invention further includes DNA encoding the ribozymes, DNA encoding ribozymes which has been inserted into an expression vector, host cells containing such vectors, and methods of decreasing QPRTase production in plants using ribozymes.

Nucleic acid sequences employed in carrying out the present invention include those with sequence similarity to **SEQ ID NO:1**, and encoding a protein having quinolate phosphoribosyl transferase activity. This definition is intended to encompass natural allelic variations in QPRTase proteins. Thus, DNA sequences that hybridize to DNA of **SEQ ID NO:1** and code for expression of QPRTase, particularly plant QPRTase enzymes, may also be employed in carrying out the present invention.

Multiple forms of tobacco QPRT enzyme may exist. Multiple forms of an enzyme may be due to post-translational modification of a single gene product, or to multiple forms of the *NtQPT1* gene.

Conditions which permit other DNA sequences which code for expression of a protein having QPRTase activity to hybridize to DNA of **SEQ ID NO:1** or to other DNA sequences encoding the protein given as **SEQ ID NO:2** can be determined in a routine manner. For example, hybridization of such sequences may be carried out under conditions of reduced stringency or even stringent conditions (e.g., conditions represented by a wash stringency of 0.3 M NaCl, 0.03 M sodium citrate, 0.1% SDS at 60°C or even 70°C to DNA encoding the protein given as **SEQ ID NO:2** herein in a standard *in situ* hybridization assay. *See* J. Sambrook et al., Molecular Cloning, A Laboratory Manual (2d Ed. 1989)(Cold Spring Harbor Laboratory)). In general, such sequences will be at least 65% similar, 75% similar, 80% similar, 85% similar, 90% similar, or even 95% similar, or more, with the sequence given herein as **SEQ ID NO:1**, or DNA sequences encoding proteins of **SEQ ID NO:2**. (Determinations of sequence similarity are made with the two sequences aligned for maximum matching; gaps in either of the two sequences being matched are allowed in maximizing matching. Gap lengths of 10 or less are preferred, gap lengths of 5 or less are more preferred, and gap lengths of 2 or less still more preferred.)

Differential hybridization procedures are available which allow for the isolation of cDNA clones whose mRNA levels are as low as about 0.05% of poly(A^+)RNA. *See* M. Conkling et al., *Plant Physiol.* **93**, 1203-1211 (1990). In brief, cDNA libraries are screened using single-stranded cDNA probes of reverse transcribed mRNA from plant tissue (e.g., roots and/or leaves). For differential screening, a nitrocellulose or nylon membrane is soaked in 5xSSC, placed in a 96 well suction manifold, 150 μL of stationary overnight culture transferred from a master plate to each well, and vacuum applied until all liquid has passed through the filter. 150 μL of denaturing solution (0.5M NaOH, 1.5 M NaCl) is placed in each well using a multiple pipetter and allowed to sit about 3 minutes. Suction is applied as above and the filter removed and neutralized in 0.5 M Tris-HCl (pH 8.0), 1.5 M NaCl. It is then baked 2 hours *in vacuo* and incubated with the relevant probes. By using nylon membrane filters and keeping master plates stored at -70°C in 7% DMSO, filters may be screened multiple times with multiple probes and appropriate clones recovered after several years of storage.

As used herein, the term 'gene' refers to a DNA sequence that incorporates (1) upstream (5') regulatory signals including the promoter, (2) a coding region specifying the product, protein or RNA of the gene, (3) downstream (3') regions including transcription termination and polyadenylation signals and (4) associated sequences required for efficient and specific expression.

The DNA sequence of the present invention may consist essentially of the sequence provided herein (**SEQ ID NO:1**), or equivalent nucleotide sequences representing alleles or polymorphic variants of these genes, or coding regions thereof.

Use of the phrase "substantial sequence similarity" in the present specification and claims means that DNA, RNA or amino acid sequences which have slight and non-consequential sequence variations from the actual sequences disclosed and claimed herein are considered to be equivalent to the sequences of the present invention. In this regard, "slight and non-consequential sequence variations" mean that "similar" sequences (i.e., the sequences that have substantial sequence similarity with the DNA, RNA, or proteins disclosed and claimed herein) will be functionally equivalent to the sequences disclosed and claimed in the present invention. Functionally equivalent sequences will function in substantially the same manner to produce substantially the same compositions as the nucleic acid and amino acid compositions disclosed and claimed herein.

DNA sequences provided herein can be transformed into a variety of host cells. A variety of suitable host cells, having desirable growth and handling properties, are readily available in the art.

Use of the phrase "isolated" or "substantially pure" in the present specification and claims as a modifier of DNA, RNA, polypeptides or proteins means that the DNA, RNA, polypeptides or proteins so designated have been separated from their *in vivo* cellular environments through the efforts of human beings.

As used herein, a "native DNA sequence" or "natural DNA sequence" means a DNA sequence which can be isolated from non-transgenic cells or tissue. Native DNA sequences are those which have not been artificially altered, such as by site-directed mutagenesis. Once native DNA sequences are identified, DNA molecules having native DNA sequences may be chemically synthesized or produced using recombinant DNA procedures as are known in the art. As used herein, a native plant DNA sequence is that which can be isolated from non-transgenic plant cells or tissue. As used herein, a native tobacco DNA sequence is that which can be isolated from non-transgenic tobacco cells or tissue.

DNA constructs, or "transcription cassettes," of the present invention include, 5' to 3' in the direction of transcription, a promoter as discussed herein, a DNA sequence as discussed herein operatively associated with the promoter, and, optionally, a termination sequence including stop signal for RNA polymerase and a polyadenylation signal for polyadenylase. All of these regulatory regions should be capable of operating in the cells of the tissue to be transformed. Any suitable termination signal may be employed in carrying out the present invention, examples thereof including, but not limited to, the nopaline synthase (*nos*) terminator, the octapine synthase (*ocs*) terminator, the CaMV terminator, or native termination signals derived from the same gene as the transcriptional initiation region or derived from a different gene. *See, e.g.*, Rezian et al. (1988) supra, and Rodermel et al. (1988), supra.

The term "operatively associated," as used herein, refers to DNA sequences on a single DNA molecule which are associated so that the function of one is affected by the other. Thus, a promoter is operatively associated with a DNA when it is capable of affecting the transcription of that DNA (i.e., the DNA is under the transcriptional control of the promoter). The promoter is said to be "upstream" from the DNA, which is in turn said to be "downstream" from the promoter.

The transcription cassette may be provided in a DNA construct which also has at least one replication system. For convenience, it is common to have a replication system functional in *Escherichia coli*, such as ColE1, pSC101, pACYC184, or the like. In this manner, at each stage after each manipulation, the resulting construct may be cloned, sequenced, and the correctness of the manipulation determined. In addition, or in place of the *E. coli* replication system, a broad host range replication system may be employed, such as the replication systems of the P-1 incompatibility plasmids, e.g., pRK290. In addition to the replication system, there will frequently be at least one marker present, which may be useful in one or more hosts, or different markers for individual hosts. That is, one marker may be employed for selection in a prokaryotic host, while another marker may be employed for selection in a eukaryotic host, particularly the plant host. The markers may be protection against a biocide, such as antibiotics, toxins, heavy metals, or the like; may provide complementation, by imparting prototrophy to an auxotrophic host; or may provide a visible phenotype through the production of a novel compound in the plant.

The various fragments comprising the various constructs, transcription cassettes, markers, and the like may be introduced consecutively by restriction enzyme cleavage of an appropriate replication system, and insertion of the particular construct or fragment into the available site. After ligation and cloning the DNA construct may be isolated for further manipulation. All of these techniques are amply exemplified in the literature as exemplified by J. Sambrook et al., Molecular Cloning, A Laboratory Manual (2d Ed. 1989)(Cold Spring Harbor Laboratory).

Vectors which may be used to transform plant tissue with nucleic acid constructs of the present invention include both *Agrobacterium* vectors and ballistic vectors, as well as vectors suitable for DNA-mediated transformation.

The term 'promoter' refers to a region of a DNA sequence that incorporates the necessary signals for the efficient expression of a coding sequence. This may include sequences to which an RNA polymerase binds but is not limited to such sequences and may include regions to which other regulatory proteins bind together with regions involved in the control of protein translation and may include coding sequences.

Promoters employed in carrying out the present invention may be constitutively active promoters. Numerous constitutively active promoters which are operable in plants are available. A preferred example is the Cauliflower Mosaic Virus (CaMV) 35S promoter which is expressed constitutively in most plant tissues. In the alternative, the promoter may be a root-specific promoter or root cortex specific promoter, as explained in greater detail below.

Antisense sequences have been expressed in transgenic tobacco plants utilizing the Cauliflower Mosaic Virus (CaMV) 35S promoter. *See, e.g.*, Cornelissen et al., "Both RNA Level and Translation Efficiency are Reduced by Anti-Sense RNA in Transgenic Tobacco", *Nucleic Acids Res.* 17, pp. 833-43 (1989); Rezaian et al., "Anti-Sense RNAs of Cucumber Mosaic Virus in Transgenic Plants Assessed for Control of the Virus", *Plant Molecular Biology* 11, pp. 463-71 (1988); Rodermel et al., "Nuclear-Organelle Interactions: Nuclear Antisense Gene Inhibits Ribulose Bisphosphate Carboxylase Enzyme Levels in Transformed Tobacco Plants", *Cell* 55, pp. 673-81 (1988); Smith et al., "Antisense RNA Inhibition of Polygalacturonase Gene Expression in Transgenic Tomatoes", *Nature* 334, pp. 724-26 (1988); Van der Krol et al., "An Anti-Sense Chalcone Synthase Gene in Transgenic Plants Inhibits Flower Pigmentation", *Nature* 333, pp. 866-69 (1988).

Use of the CaMV 35S promoter for expression of QPRTase in the transformed tobacco cells and plants of this invention is preferred. Use of the CaMV promoter for expression of other recombinant genes in tobacco roots has been well described (Lam et al., "Site-Specific Mutations Alter In Vitro Factor Binding and Change Promoter Expression Pattern in Transgenic Plants", *Proc. Nat. Acad. Sci. USA* 86, pp. 7890-94 (1989); Poulsen et al. "Dissection of 5' Upstream Sequences for Selective Expression of the Nicotiana plumbaginifolia rbcS-8B Gene", *Mol. Gen. Genet.* 214, pp. 16-23 (1988)).

Other promoters which are active only in root tissues (root specific promoters) are also particularly suited to the methods of the present invention. *See, e.g.,* US Patent No. 5,459,252 to Conkling et al.; Yamamoto et al., *The Plant Cell*, 3:371 (1991). The TobRD2 root-cortex specific promoter may also be utilized. *See, e.g.,* US Patent application SN 08/508,786, now allowed, to Conkling et al; PCT WO 9705261. All patents cited herein are intended to be incorporated herein by reference in their entirety.

The QPRTase recombinant DNA molecules and vectors used to produce the transformed tobacco cells and plants of this invention may further comprise a dominant selectable marker gene. Suitable dominant selectable markers for use in tobacco include, inter alia, antibiotic resistance genes encoding neomycin phosphotransferase (NPTII), hygromycin phosphotransferase (HPT), and chloramphenicol acetyltransferase (CAT). Another well-known dominant selectable marker suitable for use in tobacco is a mutant dihydrofolate reductase gene that encodes methotrexate-resistant dihydrofolate reductase. DNA vectors containing suitable antibiotic resistance genes, and the corresponding antibiotics, are commercially available.

Transformed tobacco cells are selected out of the surrounding population of non-transformed cells by placing the mixed population of cells into a culture medium containing an appropriate concentration of the antibiotic (or other compound normally toxic to tobacco cells) against which the chosen dominant selectable marker gene product confers resistance. Thus, only those tobacco cells that have been transformed will survive and multiply.

Methods of making recombinant plants of the present invention, in general, involve first providing a plant cell capable of regeneration (the plant cell typically residing in a tissue capable of regeneration). The plant cell is then transformed with a DNA construct comprising a transcription cassette of the present invention (as described herein) and a recombinant plant is regenerated from the transformed plant cell. As explained below, the transforming step is carried out by techniques as are known in the art, including but not limited to bombarding the plant cell with microparticles carrying the transcription cassette, infecting the cell with an *Agrobacterium tumefaciens* containing a Ti plasmid carrying the transcription cassette, or any other technique suitable for the production of a transgenic plant.

Numerous *Agrobacterium* vector systems useful in carrying out the present invention are known. For example, U.S. Patent No. 4,459,355 discloses a method for transforming susceptible plants, including dicots, with an *Agrobacterium* strain containing the Ti plasmid. The transformation of woody plants with an *Agrobacterium* vector is disclosed in U.S. Patent No. 4,795,855. Further, U.S. Patent No. 4,940,838 to Schilperoort et al. discloses a binary *Agrobacterium* vector (i.e., one in which the *Agrobacterium* contains one plasmid having the vir region of a Ti plasmid but no T region, and a second plasmid having a T region but no vir region) useful in carrying out the present invention.

Microparticles carrying a DNA construct of the present invention, which microparticle is suitable for the ballistic transformation of a plant cell, are also useful for making transformed plants of the present invention. The microparticle is propelled into a plant cell to produce a transformed plant cell, and a plant is regenerated from the transformed plant cell. Any suitable ballistic cell transformation methodology and apparatus can be used in practicing the present invention. Exemplary apparatus and procedures are disclosed in Sanford and Wolf, U.S. Patent No. 4,945,050, and in Christou et al., U.S. Patent No. 5,015,580. When using ballistic transformation procedures, the transcription cassette may be incorporated into a plasmid capable of replicating in or integrating into the cell to be transformed. Examples of microparticles suitable for use in such systems include 1 to 5 μm gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

Plant species may be transformed with the DNA construct of the present invention by the DNA-mediated transformation of plant cell protoplasts and subsequent regeneration of the plant from the transformed protoplasts in accordance with procedures well known in the art. Fusion of tobacco protoplasts with DNA-containing liposomes or via electroporation is known in the art. (Shillito et al., "Direct Gene Transfer to Protoplasts of Dicotyledonous and Monocotyledonous Plants by a Number of Methods, Including Electroporation", *Methods in Enzymology* 153, pp. 313-36 (1987)).

As used herein, transformation refers to the introduction of exogenous DNA into cells, so as to produce transgenic cells stably transformed with the exogenous DNA.

Transformed cells are induced to regenerate intact tobacco plants through application of tobacco cell and tissue culture techniques that are well known in the art. The method of plant regeneration is chosen so as to be compatible with the method of transformation. The stable presence and the orientation of the QPRTase sequence in transgenic tobacco plants can be verified by Mendelian inheritance of the QPRTase sequence, as revealed by standard methods of DNA analysis applied to progeny resulting from controlled crosses. After regeneration of transgenic tobacco plants from transformed cells, the introduced DNA sequence is readily transferred to other tobacco varieties through conventional plant breeding practices and without undue experimentation.

For example, to analyze the segregation of the transgene, regenerated transformed plants (R_0) may be grown to maturity, tested for nicotine levels, and selfed to produce R_1 plants. A percentage of R_1 plants carrying the transgene are homozygous for the transgene. To identify homozygous R_1 plants, transgenic R_1 plants are grown to maturity and selfed. Homozygous R_1 plants will produce R_2 progeny where each progeny plant carries the transgene; progeny of heterozygous R_1 plants will segregate 3:1.

As nicotine serves as a natural pesticide which helps protect tobacco plants from damage by pests. It may therefor be desirable to additionally transform low or no nicotine plants produced by the present methods with a transgene (such as *Bacillus thuringiensis*) that will confer additional insect protection.

A preferred plant for use in the present methods are species of *Nicotiana*, or tobacco, including *N. tabacum, N. rustica and N. glutinosa*. Any strain or variety of tobacco may be used. Preferred are strains that are already low in nicotine content, such as *Nic1/Nic2* double mutants.

Any plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a vector of the present invention. The term "organogenesis," as used herein, means a process by which shoots and roots are developed sequentially from meristematic centers; the term "embryogenesis," as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, callus tissue, existing meristematic tissue (e.g., apical meristems, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem).

Plants of the present invention may take a variety of forms. The plants may be chimeras of transformed cells and non-transformed cells; the plants may be clonal transformants (e.g., all cells transformed to contain the transcription cassette); the plants may comprise grafts of transformed and untransformed tissues (e.g., a transformed root stock grafted to an untransformed scion in citrus species). The transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, first generation (or T1) transformed plants may be selfed to give homozygous second generation (or T2) transformed plants, and the T2 plants further propagated through classical breeding techniques. A dominant selectable marker (such as *npt*II) can be associated with the transcription cassette to assist in breeding.

In view of the foregoing, it will be apparent that plants which may be employed in practicing the present invention include those of the genus *Nicotiana*.

Those familiar with the recombinant DNA methods described above will recognize that one can employ a full-length QPRTase cDNA molecule or a full-length QPRTase chromosomal gene, joined in the sense orientation, with appropriate operably linked regulatory sequences, to construct transgenic tobacco cells and plants. (Those of skill in the art will also recognize that appropriate regulatory sequences for expression of genes in the sense orientation include any one of the known eukaryotic translation start sequences, in addition to the promoter and polyadenylation/transcription termination sequences described above). Such transformed tobacco plants are characterized by increased levels of QPRTase, and thus by higher nicotine content than untransformed control tobacco plants.

It should be understood, therefore, that use of QPRTase DNA sequences to decrease or to increase levels of QPRT enzyme, and thereby to decrease or increase the nicotine content in tobacco plants, falls within the scope of the present invention.

As used herein, a crop comprises a plurality of plants of the present invention, and of the same genus, planted together in an agricultural field. By "agricultural field" is meant a common plot of soil or a greenhouse. Thus, the present invention provides a method of producing a crop of plants having altered QPTRase activity and thus having increased or decreased nicotine levels, compared to a similar crop of non-transformed plants of the same species and variety.

The examples which follow are set forth to illustrate the present invention, and are not to be construed as limiting thereof.

EXAMPLE 1

Isolation and Sequencing

TobRD2 cDNA (Conkling et al., *Plant Phys.* 93, 1203 (1990)) was sequenced and is provided herein as **SEQ ID NO:1,** and the deduced amino acid sequence as **SEQ ID NO:2**. The deduced amino acid sequence was predicted to be a cytosolic protein. Although plant QPTase genes have not been reported, comparisons of the *NtPT1* amino acid sequence with the GenBank database (**Figure 3**) revealed limited sequence similarity to certain bacterial and other proteins; quinolate phosphoribosyl transferase (QPRTase) activity has been demonstrated for the *S. typhimurium, E. coli.* and *N. tabacum* genes. The *NtQPT1* encoded QPTase has similarity to the deduced peptide fragment encoded by an *Arabidopsis* EST (expression sequence tag) sequence (Genbank Accession number F20096), which may represent part of an *Arabidopsis* QPTase gene.

EXAMPLE 2

In-Situ Hybridizations

To determine the spatial distribution of TobRD2 mRNA transcripts in the various tissues of the root, *in situ* hybridizations were performed in untransformed plants. In-situ hybridizations of antisense strand of TobRD2 to the TobRD2 mRNA in root tissue was done using techniques as described in Meyerowitz, *Plant Mol. Biol. Rep.* 5,242 (1987) and Smith et al., *Plant Mol. Biol. Rep.* 5, 237 (1987). Seven day old tobacco (*Nicotania tabacum*) seedling roots were fixed in phosphate-buffered glutaraldehyde, embedded in Paraplast Plus (Monoject Inc., St. Louis, MO) and sectioned at 8 mm thickness to obtain transverse as well as longitudinal sections. Antisense TobRD2 transcripts, synthesized in vitro in the presence of 35S-ATP, were used as probes. The labeled RNA was hydrolyzed by alkaline treatment to yield 100 to 200 base mass average length prior to use.

Hybridizations were done in 50% formamide for 16 hours at 42°C, with approximately 5×10^6 counts-per-minute (cpm) labeled RNA per milliliter of hybridization solution. After exposure, the slides were developed and visualized under bright and dark field microscopy.

The hybridization signal was localized to the cortical layer of cells in the roots (results not shown). Comparison of both bright and dark field images of the same sections localized TobRD2 transcripts to the parenchymatous cells of the root cortex. No hybridization signal was visible in the epidermis or the stele.

EXAMPLE 3

TobRD2 mRNA Levels in *Nic1* and *Nic2* Tobacco Mutants and Correlation to Nicotine Levels

TobRD2 steady-state mRNA levels were examined in *Nic1* and *Nic2* mutant tobacco plants. *Nic1* and *Nic2* are known to regulate quinolate phosphoribosyl transferase activity and putrescence methyl-transferase activity, and are co-dominant regulators of nicotine production. The present results are illustrated in **Figures 5A** and **5B** show that *TobRD2* expression is regulated by *Nic1* and *Nic2*.

RNA was isolated from the roots of wild-type Burley 21 tobacco plants (*Nic1/Nic1 Nic2/Nic2*); roots of *Nic1-* Burley 21 (*nic1/nic1 Nic2/Nic2*); roots of *Nic2-* Burley 21 (*Nic1/Nic1 nic2/nic2*); and roots of *Nic1-Nic2-* Burley 21 (*nic1/nic1 nic2/nic2*).

Four Burley 21 tobacco lines (nic) were grown from seed in soil for a month and transferred to hydroponic chambers in aerated nutrient solution in a greenhouse for one month. These lines were isogenic, except for the two low-nicotine loci, and had genotypes of *Nic1/Nic1 Nic2/Nic2, Nic1/Nic1 nic2/nic2, nic1/nic1 Nic2/Nic2, nic1/nic1 nic2/nic2*. Roots were harvested from about 20 plants for each genotype and pooled for RNA isolation. Total RNA (1μg) from each genotype was electrophoresed through a 1% agarose gel containing 1.1M formaldehyde and transferred to a nylon membrane according to Sambrook et al. (1989). The membranes were hybridized with ^{32}P-labeled TobRD2 cDNA fragments. Relative intensity of TobRD2 transcripts were measured by densitometry. **Figure 5** (solid bars)

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Conkling, Mark A.
Mendu, Nandini
Song, Wen

(ii) TITLE OF INVENTION: Regulation of Quinolate Phosphoribosyl Transferase Expression

(iii) NUMBER OF SEQUENCES: 4

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Sibley, Kenneth D.
(B) REGISTRATION NUMBER: 31,665
(C) REFERENCE/DOCKET NUMBER: 5051-338P

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 919-420-2200
(B) TELEFAX: 919-881-3175

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1399 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 52..1104

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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                                                         Met Phe
                                                           1

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Arg Ala Ile Pro Phe Thr Ala Thr Val His Pro Tyr Ala Ile Thr Ala
         5                  10                  15

CCA AGG TTG GTG GTG AAA ATG TCA GCA ATA GCC ACC AAG AAT ACA AGA     153
Pro Arg Leu Val Val Lys Met Ser Ala Ile Ala Thr Lys Asn Thr Arg
     20                  25                  30

GTG GAG TCA TTA GAG GTG AAA CCA CCA GCA CAC CCA ACT TAT GAT TTA     201
Val Glu Ser Leu Glu Val Lys Pro Pro Ala His Pro Thr Tyr Asp Leu
 35                  40                  45                  50

AAG GAA GTT ATG AAA CTT GCA CTC TCT GAA GAT GCT GGG AAT TTA GGA     249
Lys Glu Val Met Lys Leu Ala Leu Ser Glu Asp Ala Gly Asn Leu Gly
                 55                  60                  65

GAT GTG ACT TGT AAG GCG ACA ATT CCT CTT GAT ATG GAA TCC GAT GCT     297
Asp Val Thr Cys Lys Ala Thr Ile Pro Leu Asp Met Glu Ser Asp Ala
             70                  75                  80

CAT TTT CTA GCA AAG GAA GAC GGG ATC ATA GCA GGA ATT GCA CTT GCT     345
His Phe Leu Ala Lys Glu Asp Gly Ile Ile Ala Gly Ile Ala Leu Ala
         85                  90                  95

GAG ATG ATA TTC GCG GAA GTT GAT CCT TCA TTA AAG GTG GAG TGG TAT     393
Glu Met Ile Phe Ala Glu Val Asp Pro Ser Leu Lys Val Glu Trp Tyr
    100                 105                 110

GTA AAT GAT GGC GAT AAA GTT CAT AAA GGC TTG AAA TTT GGC AAA GTA     441
Val Asn Asp Gly Asp Lys Val His Lys Gly Leu Lys Phe Gly Lys Val
115                 120                 125                 130

CAA GGA AAC GCT TAC AAC ATT GTT ATA GCT GAG AGG GTT GTT CTC AAT     489
Gln Gly Asn Ala Tyr Asn Ile Val Ile Ala Glu Arg Val Val Leu Asn
                135                 140                 145

TTT ATG CAA AGA ATG AGT GGA ATA GCT ACA CTA ACT AAG GAA ATG GCA     537
Phe Met Gln Arg Met Ser Gly Ile Ala Thr Leu Thr Lys Glu Met Ala
            150                 155                 160

GAT GCT GCA CAC CCT GCT TAC ATC TTG GAG ACT AGG AAA ACT GCT CCT     585
Asp Ala Ala His Pro Ala Tyr Ile Leu Glu Thr Arg Lys Thr Ala Pro
        165                 170                 175
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GGA TTA CGT TTG GTG GAT AAA TGG GCG GTA TTG ATC GGT GGG GGG AAG 633
Gly Leu Arg Leu Val Asp Lys Trp Ala Val Leu Ile Gly Gly Gly Lys
180 185 190

AAT CAC AGA ATG GGC TTA TTT GAT ATG GTA ATG ATA AAA GAC AAT CAC 681
Asn His Arg Met Gly Leu Phe Asp Met Val Met Ile Lys Asp Asn His
195 200 205 210

ATA TCT GCT GCT GGA GGT GTC GGC AAA GCT CTA AAA TCT GTG GAT CAG 729
Ile Ser Ala Ala Gly Gly Val Gly Lys Ala Leu Lys Ser Val Asp Gln
215 220 225

TAT TTG GAG CAA AAT AAA CTT CAA ATA GGG GTT GAG GTT GAA ACC AGG 777
Tyr Leu Glu Gln Asn Lys Leu Gln Ile Gly Val Glu Val Glu Thr Arg
230 235 240

ACA ATT GAA GAA GTA CGT GAG GTT CTA GAC TAT GCA TCT CAA ACA AAG 825
Thr Ile Glu Glu Val Arg Glu Val Leu Asp Tyr Ala Ser Gln Thr Lys
245 250 255

ACT TCG TTG ACT AGG ATA ATG CTG GAC AAT ATG GTT GTT CCA TTA TCT 873
Thr Ser Leu Thr Arg Ile Met Leu Asp Asn Met Val Val Pro Leu Ser
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AAC GGA GAT ATT GAT GTA TCC ATG CTT AAG GAG GCT GTA GAA TTG ATC 921
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325 330 335

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340 345 350

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CATTTTACTA GTTGTCAAAC AAAAGATCCT TCACTGTGTA ATCAAACAAA AAGATGTAAA 1234

TTGCTGGAAT ATCTCAGATG GCTCTTTTCC AACCTTATTG CTTGAGTTGG TAATTTCATT 1294

ATAGCTTTGT TTTCATGTTT CATGGAATTT GTTACAATGA AAATACTTGA TTTATAAGTT 1354

TGGTGTATGT AAAATTCTGT GTTACTTCAA ATATTTTGAG ATGTT 1399

Thr Arg Thr Ile Glu Glu Val Arg Glu Val Leu Asp Tyr Ala Ser Gln
245 250 255

Thr Lys Thr Ser Leu Thr Arg Ile Met Leu Asp Asn Met Val Val Pro
260 265 270

Leu Ser Asn Gly Asp Ile Asp Val Ser Met Leu Lys Glu Ala Val Glu
275 280 285

Leu Ile Asn Gly Arg Phe Asp Thr Glu Ala Ser Gly Asn Val Thr Leu
290 295 300

Glu Thr Val His Lys Ile Gly Gln Thr Gly Val Thr Tyr Ile Ser Ser
305 310 315 320

Gly Ala Leu Thr His Ser Val Lys Ala Leu Asp Ile Ser Leu Lys Ile
325 330 335

Asp Thr Glu Leu Ala Leu Glu Val Gly Arg Arg Thr Lys Arg Ala
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1053 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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AAACCACCAG CACACCCAAC TTATGATTTA AAGGAAGTTA TGAAACTTGC ACTCTCTGAA 180

GATGCTGGGA ATTTAGGAGA TGTGACTTGT AAGGCGACAA TTCCTCTTGA TATGGAATCC 240

GATGCTCATT TTCTAGCAAA GGAAGACGGG ATCATAGCAG GAATTGCACT TGCTGAGATG 300

ATATTCGCGG AAGTTGATCC TTCATTAAAG GTGGAGTGGT ATGTAAATGA TGGCGATAAA 360

GTTCATAAAG GCTTGAAATT TGGCAAAGTA CAAGGAAACG CTTACAACAT TGTTATAGCT 420

GAGAGGGTTG TTCTCAATTT TATGCAAAGA ATGAGTGGAA TAGCTACACT AACTAAGGAA 480

ATGGCAGATG CTGCACACCC TGCTTACATC TTGGAGACTA GGAAAACTGC TCCTGGATTA 540

CGTTTGGTGG ATAAATGGGC GGTATTGATC GGTGGGGGGA AGAATCACAG AATGGGCTTA 600

TTTGATATGG TAATGATAAA AGACAATCAC ATATCTGCTG CTGGAGGTGT CGGCAAAGCT 660

CTAAAATCTG TGGATCAGTA TTTGGAGCAA AATAAACTTC AAATAGGGGT TGAGGTTGAA 720

ACCAGGACAA TTGAAGAAGT ACGTGAGGTT CTAGACTATG CATCTCAAAC AAAGACTTCG 780

TTGACTAGGA TAATGCTGGA CAATATGGTT GTTCCATTAT CTAACGGAGA TATTGATGTA 840

TCCATGCTTA AGGAGGCTGT AGAATTGATC AATGGGAGGT TTGATACGGA GGCTTCAGGA 900

AATGTTACCC TTGAAACAGT ACACAAGATT GGACAAACTG GTGTTACCTA CATTTCTAGT 960

GGTGCCCTGA CGCATTCCGT GAAAGCACTT GACATTTCCC TGAAGATCGA TACAGAGCTC 1020

GCCCTTGAAG TTGGAAGGCG TACAAAACGA GCA 1053

<u>That which is claimed is:</u>

1. An isolated DNA molecule comprising a sequence selected from the group consisting of:

(a) SEQ ID NO:1;

(b) DNA sequences which encode an enzyme having SEQ ID NO:2;

(c) DNA sequences which hybridize to isolated DNA of (a) or (b) above and which encode a quinolate phosphoribosyl transferase enzyme; and

(d) DNA sequences which differ from the DNA of (a), (b) or (c) above due to the degeneracy of the genetic code.

2. A DNA construct comprising an expression cassette, which construct comprises, in the 5' to 3' direction, a promoter operable in a plant cell and a DNA segment according to claim 1 positioned downstream from said promoter and operatively associated therewith.

3. A DNA construct comprising an expression cassette, which construct comprises, in the 5' to 3' direction, a plant promoter and a DNA segment according to claim 1 positioned downstream from said promoter and operatively associated therewith, said DNA segment in antisense orientation.

4. A DNA construct comprising, in the 5' to 3' direction, a promoter operable in a plant cell and DNA encoding a plant quinolate phosphoribosyl transferase, said DNA operably associated with said promoter.

5. A DNA construct comprising, in the 5' to 3' direction, a promoter operable in a plant cell and DNA encoding a plant quinolate phosphoribosyl transferase, said DNA in antisense orientation and operably associated with said promoter.

20. The method of claim 16, further comprising regenerating a plant from said transformed plant cell.

21. A method according to claim 16, wherein said promoter is constitutively active.

22. A method according to claim 16, wherein said promoter is selectively active in plant root tissue cells.

23. A method according to claim 16, wherein said promoter is selectively active in plant root cortex tissue cells.

24. A method according to claim 16, wherein said transforming step is carried out by bombarding said plant cell with microparticles carrying said DNA construct.

25. A method according to claim 16 wherein said transforming step is carried out by infecting said plant cell with an *Agrobacterium tumefaciens* containing a Ti plasmid carrying said DNA construct.

26. A method of producing transgenic tobacco seeds, comprising collecting seed from a transgenic tobacco plant produced by the method of claim 19.

27. The method according to claim 16, wherein said exogenous DNA sequence is complementary to said quinolate phosphoribosyl transferase messenger RNA (QPRT mRNA) expressed in said plant cell in a region selected from:

(a) the 5'-untranslated sequence of said QPRT mRNA;

(b) the 3'-untranslated sequence of said QPRT mRNA; and

(c) the translated region of said QPRT mRNA.

28. The method according to claim 16, wherein said exogenous DNA sequence is complementary to at least 15 nucleotides of said quinolate phosphoribosyl transferase messenger RNA expressed in said plant cell

29. The method according to claim16, wherein said exogenous DNA sequence is complementary to at least 200 nucleotides of said quinolate phosphoribosyl transferase messenger RNA expressed in said plant cell

30. The method according to claim 16, wherein said exogenous DNA sequence comprises a quinolate phosphoribosyl transferase encoding sequence selected from the DNA sequences of Claim 1.

31. A transgenic plant of the species *Nicotiana* having reduced quinolate phosphoribosyl transferase (QPRTase) expression relative to a non-transformed control plant, said transgenic plant comprising transgenic plant cells containing:

an exogenous DNA construct comprising, in the 5' to 3' direction, a promoter operable in said plant cell and DNA comprising a segment of a DNA sequence that encodes a plant quinolate phosphoribosyl transferase mRNA, said DNA operably associated with said promoter;

said plant exhibiting reduced QPRTase expression compared to a non-transformed control plant.

32. The method of claim 31, wherein said segment of DNA comprising a segment of a DNA sequence encoding quinolate phosphoribosyl transferase mRNA is in antisense orientation.

33. The method of claim 31, wherein said segment of DNA comprising a segment of a DNA sequence encoding quinolate phosphoribosyl transferase mRNA is in sense orientation.

growing a plant cell transformed to contain exogenous DNA, wherein said exogenous DNA encodes quinolate phosphoribosyl transferase.

43. The method according to claim 83, wherein said transformed plant cell is obtained by a method comprising:

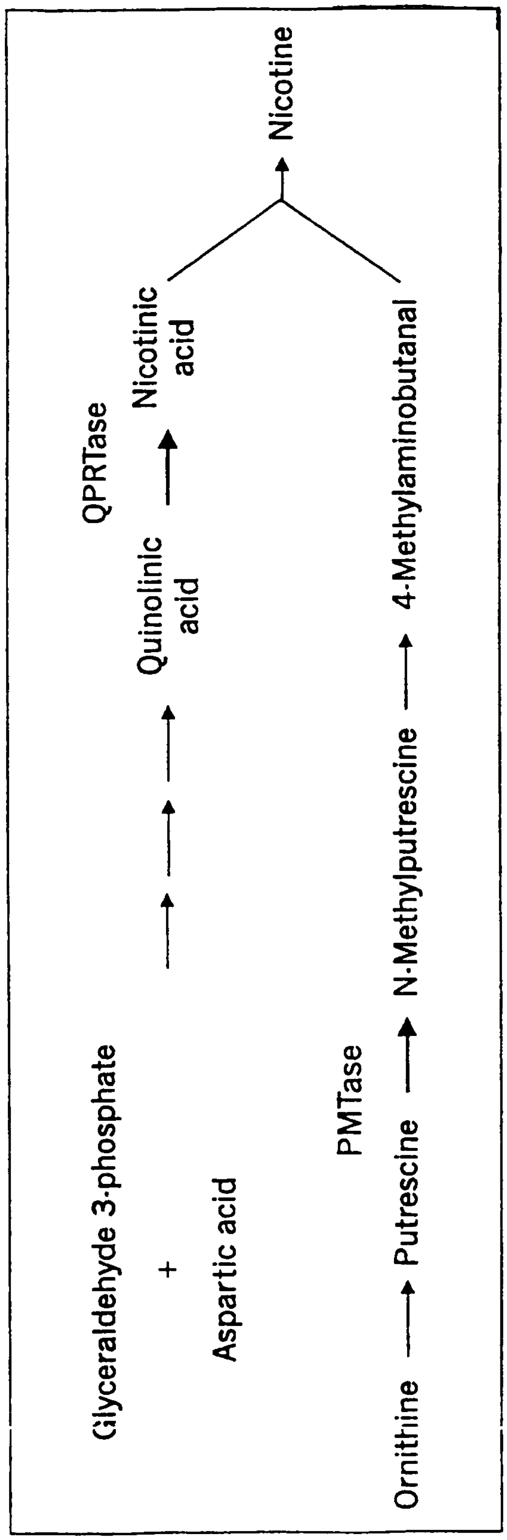
integrating into the genome of a host plant cell a construct comprising, in the direction of transcription, a promoter functional in said plant cell, a DNA sequence encoding quinolate phosphoribosyl transferase functional in said cell, said DNA sequence operably associated with said promoter, and a transcriptional termination region functional in said cell, whereby a transformed plant cell is obtained.

44. A method of producing a tobacco plant having increased levels of nicotine in leaves of said tobacco plant, said method comprising:

growing a tobacco plant, or progeny plants thereof, wherein said plant comprises cells containing a DNA construct comprising a transcriptional initiation region functional in said plant and an exogenous DNA sequence operably joined to said transcriptional initiation region, wherein said DNA sequence encodes quinolate phosphoribosyl transferase functional in said cells.

* * *

FIG. 1


Glyceraldehyde 3-phosphate
+
Aspartic acid
Quinolinic acid
QPRTase
Nicotinic acid
Nicotine
Ornithine
Putrescine
PMTase
N-Methylputrescine
4-Methylaminobutanal

```
caaaaactat tttccacaaa attcatttca caacccccccc aaaaaaaaac cATGTTTAGA   60
GCTATTCCTT TCACTGCTAC AGTGCATCCT TATGCAATTA CAGCTCCAAG GTTGGTGGTG  120
AAAATGTCAG CAATAGCCAC CAAGAATACA AGAGTGGAGT CATTAGAGGT GAAACCACCA  180
GCACACCCAA CTTATGATTT AAAGGAAGTT ATGAAACTTG CACTCTCTGA AGATGCTGGG  240
TTTCTAGCAA AGGAAGACGG GATCATAGCA GGAATTGCAC TTGCTGAGAT GATATTCGCG  360
GAAGTTGATC CTTCATTAAA GGTGGAGTGG TATGTAAATG ATGGCGATAA AGTTCATAAA  420
GGCTTGAAAT TTGGCAAAGT ACAAGGAAAC GCTTACAACA TTGTTATAGC TGAGAGGGTT  480
GTTCTCAATT TTATGCAAAG AATGAGTGGA ATAGCTACAC TAACTAAGGA AATGGCAGAT  540
GCTGCACACC CTGCTTACAT CTTGGAGACT AGGAAAACTG CTCCTGGATT ACGTTTGGTG  600
GATAAATGGG CGGTATTGAT CGGTGGGGGG AAGAATCACA GAATGGGCTT ATTTGATATG  660
GTAATGATAA AAGACAATCA CATATCTGCT GCTGGAGGTG TCGGCAAAGC TCTAAAATCT  720
GTGGATCAGT ATTTGGAGCA AAATAAACTT CAAATAGGGG TTGAGGTTGA AACCAGGACA  780
ATTGAAGAAG TACGTGAGGT TCTAGACTAT GCATCTCAAA CAAAGACTTC GTTGACTAGG  840
ATAATGCTGG ACAATATGGT TGTTCCATTA TCTAACGGAG ATATTGATGT ATCCATGCTT  900
AAGGAGGCTG TAGAATTGAT CAATGGGAGG TTTGATACGG AGGCTTCAGG AAATGTTACC  960
CTTGAAACAG TACACAAGAT TGGACAAACT GGTGTTACCT ACATTTCTAG TGGTGCCCTG 1020
ACGCATTCCG TGAAAGCACT TGACATTTCC CTGAAGATCG ATACAGAGCT CGCCCTTGAA 1080
GTTGGAAGGC GTACAAAACG AGCATGAgcg ccattacttc tgctataggg ttggagtaaa 1140
agcagctgaa tagctgaaag gtgcaaataa gaatcatttt actagttgtc aaacaaaaga 1200
tccttcactg tgtaatcaaa caaaaagatg taaattgctg gaatatctca gatggctctt 1260
ttccaacctt attgcttgag ttggtaattt cattatagct ttgttttcat gtttcatgga 1320
atttgttaca atgaaaatac ttgatttata agtttggtgt atgtaaaatt ctgtgttact 1380
tcaaatattt tgagatgtt                                               1399
```

FIGURE 2A

```
MFRAIPFTAT VHPYAITAPR LVVKMSAIAT KNTRVESLEV KPPAHPTYDL   50
KEVMKLALSE DAGNLGDVTC KATIPLDMES DAHFLAKEDG IIAGIALAEM  100
IFAEVDPSLK VEWYVNDGDK VHKGLKFGKV QGNAYNIVIA ERVVLNFMQR  150
MSGIATLTKE MADAAHPAYI LETRKTAPGL RLVDKWAVLI GGGKNHRMGL  200
FDMVMIKDNH ISAAGGVGKA LKSVDQYLEQ NKLQIGVEVE TRTIEEVREV  250
LDYASQTKTS LTRIMLDNMV VPLSNGDIDV SMLKEAVELI NGRFDTEASG  300
NVTLETVHKI GQTGVTYISS GALTHSVKAL DISLKIDTEL ALEVGRRTKR  350
A                                                       351
```

FIGURE 2B

```
N. tabacum      MFRAIPFTATVHPYAITAPRLVVKMSAIATKNTRVESLEVKPPAHPTYDL
R. rubrum       *--------RPNH----------------------PVAALS*F----AI
M. leprae       *--------LSDC----------------------EFDAAR--------
S. typhimurium  *--------PPRR*NPDDR*-----------DALL*RINLDI*A----AV
E. coli         *--------PPRR*NPDTR*-----------DELL*RINLDI*G----AV
H. sapien       *---------------D*EG*ALLLPPVTLAALVDSWLREDC*G------
S. cerevisiae   *---------------PVYE-HLLPVNGAWRQDVTNWLSEDV*S------

N. tabacum      KEVMKLALSEDAGNLGDVTCKATIPLDMESDAHFLAKEDGIIAGIA----
R. rubrum       D*AVRR**A**L*RA**I*ST****AATRAH*RFV*RQP**L**LGCA--
M. leprae       -DTIRR**H**LRYGL*I*TQ**V*AGTVVTGSMVPR*P*VIAGVDVALL
S. typhimurium  AQALREDLGGEVDAGN*I*AQL-L*A*TQAH*TVITR*D*VF----CGKR
E. coli         AQALREDLGGTVDANN*I*A*L-L*ENSR*H*TVITR*N*VF----CGKR
H. sapien       -----------------LNYAALVSGAGP*QAALWAKSP*VL----AGQP
S. cerevisiae   -----------------FDFGGYVVGSDLKEANLYCKQD*ML----CGVP

N. tabacum      -LAEMIFAEVDPSLKVEWYVNDGDKVHKGLK------FGKVQGNAYNIVI
R. rubrum       --RSAF-ALLDDTVTFTTPLE**AEIAA*QT------VAE*A*A*RT*LA
M. leprae       VLD*VF-GVDGYRVLY--R*E**ARLQS*QP------LLTVQAA*RGLLT
S. typhimurium  WVE*VFIQLAGDDVRLT*H*D***AI*ANQT------VFELN*PARVLLT
E. coli         WVE*VFIQLAGDDVTII*H*D***VINANQS------LFELE*PSRVLLT
H. sapien       FFDAIFTQL---NCQVS*FLPE*S*LVPVAR------VAEVR*P*HDLLL
S. cerevisiae   FAW*VFNQC---ELQVE*LFKE*SFLEPSKNDSGKIVVAKIT*P*K**LL

N. tabacum      AERVVLNFMQRMSGIATLTKEMAD--AAH--PAYILETRKTAPGLRLVDK
R. rubrum       ***TA***LGHL*****R*RRFG*AI*HT--R*RLTC****T****GLE*
M. leprae       ***TM***VCHM*****V*VAWV*AVRGT--K*KIRD****L****ALQ*
S. typhimurium  G**TA***V*TL**VASEVRRYVGLL*GT--QTQL*D****L****TAL*
E. coli         G*PTA***V*TL**VASKVRHYVELLEGT--NTQL*D****L****SAL*
H. sapien       G***A**TLARC****SAAAAAVEAARGAGWTGHVAG****T**F***E*
S. cerevisiae   ***TA**ILSRS****TASHKIISLARSTGYKGTIAG****T****RLE*

N. tabacum      WAVLIGGGKNHRMGLFDMVMIKDNHISAAGGVGKALKSVDQYLEQNKLQI
R. rubrum       Y**RC***S***F**D*A*L******AVA***SA**SRAR-AGVGHMVRI
M. leprae       Y**RV***V***L**G*TAL*****VA*V*S*VD**RA*R-AAAPEL-PC
S. typhimurium  Y***C***A***L**T*AFL******I*S*S*RQ*VEKAF-W*HPD-APV
E. coli         Y***C***A***L**S*AFL******I*S*S*RQ*VEKAS-W*HPD-APV
H. sapien       YGL*V**AAS**YD*GGLVML*D**VVPP***EK*VRAARQ---AADFAL
S. cerevisiae   YSM*V**CDT**YD*SS**ML*D***W*T*SITN*V*NARA---VCGFAV
```

FIGURE 3

```
N. tabacum       GVEVETRTIEEVREVLDYASQTKTSLTRIMLDNMVVPLSNGDIDVSMLKE
R. rubrum        EI****--L*QLA***AVGGAEV-----VL****-----DAPT----*TR
M. leprae        E****S--L*QLDAM*A-EEPEL-----*L***F--*VWQTQV----AVQ
S. typhimurium   E****N--LDELDDA*K-*GADI-----*****F-----NTDQ----MR*
E. coli          E****N--L**LD*A*K-*GADI-----*****F-----ETEQ----MR*
H. sapien        K****CSSLQ**VQAAE-*GADL-----VL***F------KPEELHPTAT
S. cerevisiae    KI***CLSED*AT*AIE-*GADV-----*****F------KGDGLK*CAQ
```

```
N. tabacum       AVELI---NGRFDTEASGNVTLETVHKIG-QTGVTYISSGALTHSVKALD
R. rubrum        **DMV---A**LV*****G*S*D*IAALA-ES**D***V*******TT**
M. leprae        RRDIR---APTVLL*S**GLS**NAAIYA-G***DYLAV*******RI**
S. typhimurium   **KRV---**QARL*V*****AE*LREFA-E***DF**VG******R***
E. coli          **KRT---**KALL*V*****DK*LREFA-E***DF**V*******Q***
H. sapien        *LKAQFPSVA---VEA**GIT*DNLPQF-CGPHIDV**M*M**QA*P***
S. cerevisiae    SLKNKWNGKKHFLLEC**GLN*DNLEEYLCD-DIDIY*TSSIHQGTPVI*
```

		% Identity	% Similarity
N. tabacum	ISKLIDTELALEVGRRTKRA		
R. rubrum	*G*D*VVA-----PPKAERA	15.9	43.2
M. leprae	*G*DL	18.3	37.3
S. typhimurium	LSMRFC	18.2	34.8
E. coli	LSMRFR	17.9	32.8
H. sapien	F***L---F*K*VAPVP*IH	16.8	31.7
S. cerevisiae	F***LAH	14.6	27.8

FIGURE 3 continued

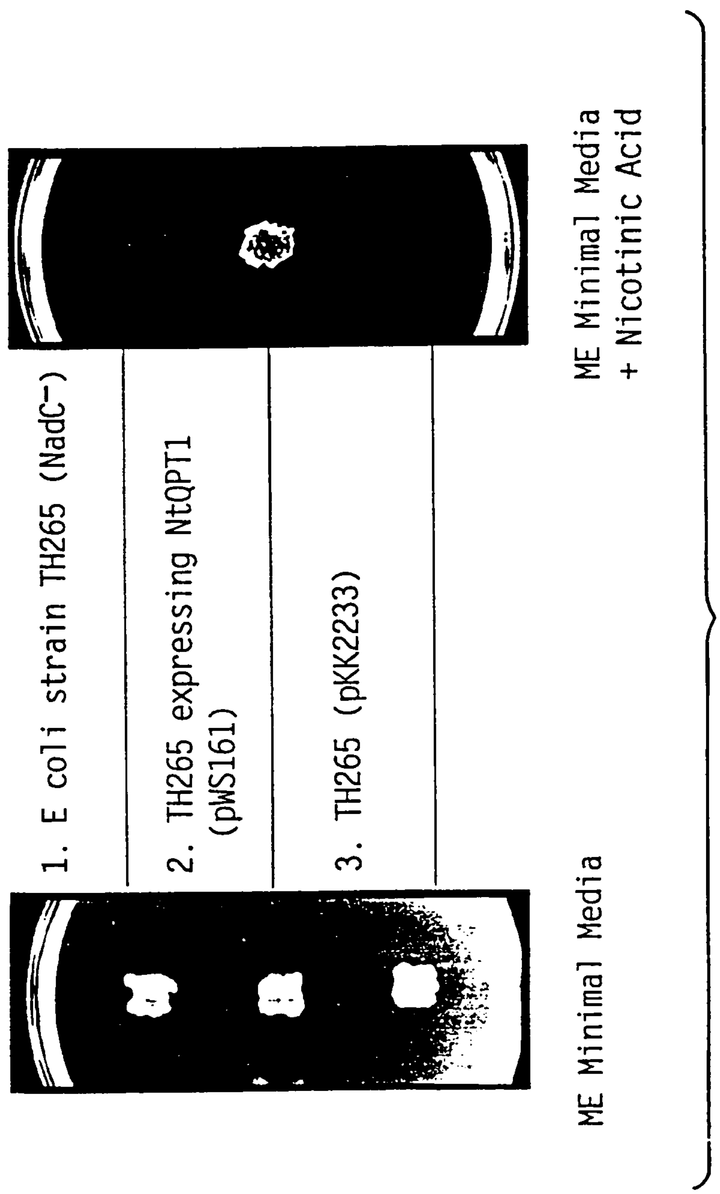

1. E coli strain TH265 (NadC−)
2. TH265 expressing NtQPT1 (pWS161)
3. TH265 (pKK2233)
ME Minimal Media
ME Minimal Media + Nicotinic Acid

FIG.4

FIG. 5

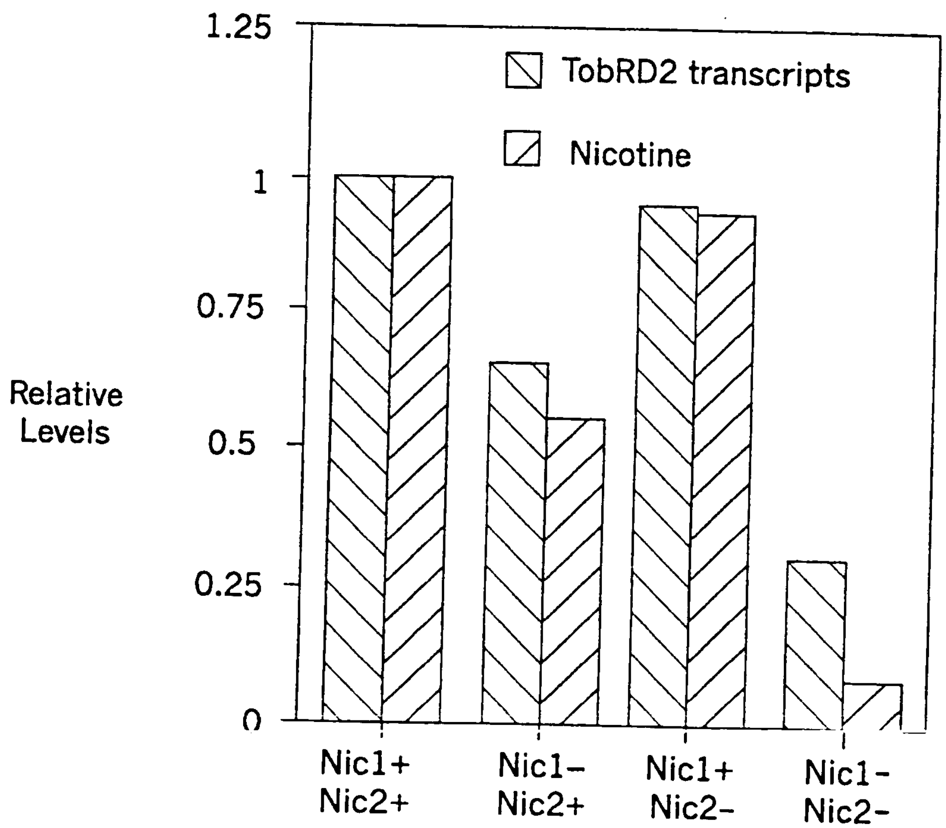
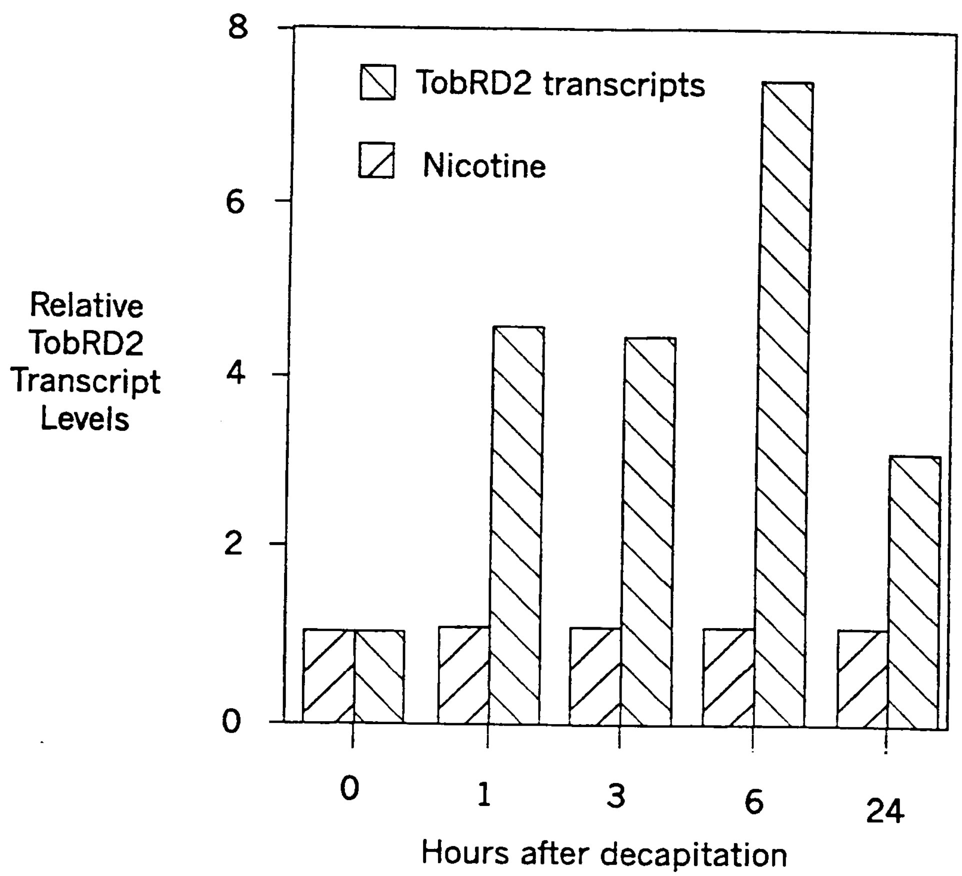

1.25
1
0.75
0.5
0.25
0
Relative Levels
TobRD2 transcripts
Nicotine
Nic1+ Nic2+
Nic1- Nic2+
Nic1+ Nic2-
Nic1- Nic2-

FIG. 6


8
6
4
2
0
TobRD2 transcripts
Nicotine
Relative TobRD2 Transcript Levels
0
1
3
6
24
Hours after decapitation

INTERNATIONAL SEARCH REPORT

Intern ial Application No
PCT/US 98/11893

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/54 C12N15/82 C12N15/70 C12N15/11 C12N9/10
C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CONKLING M. ET AL.: "Isolation of transcriptionally regulated root-specific genes from tobacco" PLANT PHYSIOLOGY, vol. 93, no. 3, July 1990, pages 1203-1211, XP002080227 cited in the application see the whole document ---	1,14,15
X	SONG, WEN: "Molecular characterizations of two tobacco root-specific genes: TobRB7 and NtQPT1." (1997) 224 PP. AVAIL.: UMI, ORDER NO. DA9804246 FROM: DISS. ABSTR. INT., B 1998, 58(8), 4061, XP002080228 see abstract ---	1-15

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 October 1998	22/10/1998

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

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PCT/US 98/11893

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HAMILL J D ET AL: "Over-expressing a yeast ornithine decarboxylase gene in transgenic roots of Nicotiana rustica can lead to enhanced nicotine accumulation." PLANT MOLECULAR BIOLOGY, (1990 JUL) 15 (1) 27-38. JOURNAL CODE: A60. ISSN: 0167-4412., XP002080229 see the whole document ---	1-44
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Intern al Application No
PCT/US 98/11893

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